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SOME FACTORS INFLUENCING TOXIN PRODUCTION AND ACCUMULATION BY *Anabaena flos-aquae* NRC-44-1

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled SOME FACTORS INFLUENCING TOXIN PRODUCTION AND ACCUMULATION BY *Anabaena flos-aquae* NRC-44-1, submitted by Wayne William Carmichael in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

Outbreaks of algal poisoning are caused by a combination of genetic and environmental factors. Under some, as yet poorly defined, environmental conditions, toxic strains of *Anabaena flos-aquae* (Lyngb.) de Bréb. may become dominant in a waterbloom and cause significant amounts of a toxin, called Very Fast Death Factor (VFDF) to accumulate. Death of livestock or other animals may occur if they consume enough of such a bloom to equal or exceed a critical dose. Previous work with a toxic clone (NRC-44-1) from Burton Lake, Saskatchewan indicated that toxicity depends on such factors as age, light, temperature and lysis. This thesis examines the roles that oxygen tension, mineral nutrition and bacterial contaminants play on toxin production and accumulation by the toxic clone NRC-44-1.

Oxygen tension was found not to stimulate toxin production in *A. flos-aquae*. Low oxygen tensions such as occur in decomposing blooms, tended to reduce rather than increase specific toxicity in nonaxenic cultures. Results of the mineral nutrient experiments did not reveal any large stimulation or suppression of toxicity caused by specific elements. It appears that only great differences in the nutrient levels of water, sufficient to inhibit growth of *A. flos-aquae*, are likely to affect toxicity of blooms.

An improved method was developed to repurify NRC-44-1 which was a modification of the technique originally used by Kim and Gorham. The technique involved washing and plating in agar plus mineral and organic media to isolate the alga and allow its separation from bacteria. An important step in the purification procedure was the use of phenol in darkness which greatly improved the selective killing of most of the bacteria and allowed algal growth to continue.

Axenic NRC-44-1 was found to have a higher specific toxicity than nonaxenic NRC-44-1 grown under the same physical conditions. This indicated that bacteria were responsible for degradation or detoxification of some of the VFDF produced, and accounted for variable toxicity observed in the laboratory. It is suggested that bacteria may account for some of the variability in toxicity of waterblooms observed in nature.

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INTRODUCTION

The problem of toxic blue-green algae is mainly one of economic loss rather than one of concern for human health. Algal poisonings have periodically caused the death of valuable livestock and wildlife. However the generally repulsive nature of blue-green algal blooms has deterred man from ingesting sufficient quantities to cause illness or death (Gorham, 1964 b).

Of the few species of blue-green algae that have been found to include toxic strains the most important is the freshwater planktonic form *Anabaena flos-aquae* (Lyngb.) de Bréb. which produces a toxin called the Very Fast Death Factor (VFDF) (Gorham, McLachlan, Hammer and Kim, 1964). Poisonings caused by waterblooms of this species have occurred only occasionally and in various parts of the world (Schwimmer and Schwimmer, 1967). Toxicities have varied considerably from one outbreak to the next, both in degree and in length of time they have persisted (Gorham, *et al.*, 1964).

So far, four factors have been found to account for this variability in toxicity: dominance by toxic strains, detoxification by adsorption to sediments, grazing by protozoa, and growth and development of the alga in relation to light, temperature and aeration (Gorham, 1962, and Gorham, personal communication). Other factors which might be involved in the toxicity of a bloom are mineral nutrition, oxygen tension, and interactions between the algal and bacterial components.

Mineral nutrition might affect toxicity directly by stimulating or inhibiting VFDF synthesis or it might affect it indirectly through growth and development. The low oxygen tensions of decomposing waterblooms might affect VFDF synthesis or promote leakage from the cells into the surrounding water. The large numbers of bacteria which occur in waterblooms might have significant effects upon toxicity depending on the species as well as the numbers which are present.

The purpose of this investigation was to study the effects of mineral nutrition, oxygen tension, and bacterial contaminants upon production and accumulation of VFDF using the toxic unialgal clone *A. flos-aquae* NRC-44-1. The results will be interpreted in relation to the variable toxicity of *Anabaena* waterblooms in nature. The investigation also included the development of an improved modification of Kim and Gorham's method for obtaining an axenic culture of the toxic clone and comparisons of toxicity in both the axenic and nonaxenic condition.

LITERATURE REVIEW

A. Algae Responsible for Toxic Blooms

Physiological and environmental examination of toxin production in blue-green algae has had to wait until specific toxin-producing strains were isolated in unialgal or axenic culture. The five genera of blue-greens most implicated in algal poisonings include *Microcystis*, *Aphanizomenon*, *Anabaena*, *Nodularia*, and *Gloeotrichia*. The species which have received the most attention because they have been the most frequent offenders include *Microcystis aeruginosa* Kütz. emend. Elenkin, *Aphanizomenon flos-aquae* (L.) Ralfs. and *Anabaena flos-aquae* (Lyngb.) de Bréb. (Grant and Hughes, 1953).

One of the first persons in North America to isolate and grow a unialgal toxic strain of *M. aeruginosa* was Olson (1951). Toxicity of the isolate varied from culture to culture (650 to 3400 mg dry weight per kg body weight) and correlation with toxic blooms was difficult because no symptoms were reported. Later work by Hughes, Gorham and Zehnder (1958) resulted in a more toxic colony isolate of *Microcystis* from a waterbloom in Little Rideau Lake, Ontario. Depending on culture conditions and treatments, the cells of *M. aeruginosa* NRC-1 had a minimum lethal dosage (MLD) or LD_{100} ranging from 40 to greater than 480 mg/kg body weight. These figures were based on intraperitoneal injection (IP) into male white mice weighing 20 to 30 grams. There appeared to be two types of toxin present; one, termed the Fast Death Factor (FDF), caused

death in two to three hours and was shown to be algal in origin, and the other, termed Slow Death Factor (SDF), caused death in four to twenty-four hours and was bacterial in origin. Isolation and identification of the endotoxin showed it to be a cyclic polypeptide containing seven amino acids with an LD_{50} (IP) of the pure toxin for mice of 0.466 ± 0.013 mg/kg body weight (Bishop, Anet and Gorham, 1959). More recent work on the identification and isolation of the toxin of *M. aeruginosa* NRC-1 (Rama Murthy and Capindale, 1970) indicate similar symptoms but a different structure for the extracted toxin. Using more refined isolation and purification methods, seven more amino acids were found to be present in the toxin which had an LD_{100} (IP) of 0.1 mg/kg body weight using 20 gram mice. Symptoms and pathology of the toxin produced by *M. aeruginosa* NRC-1 for domestic and laboratory animals were described by Konst, McKercher, Gorham, Robertson and Howell (1965).

The first attempts to isolate strains of *Aphanizomenon flos-aquae* from both toxic and nontoxic blooms were unsuccessful (Gorham, 1965). Sawyer, Gentile and Sasner (1968) found that toxic *Aphanizomenon* blooms were present in three New Hampshire lakes. In 1964 moderate fish kills were noted from a toxic *Aphanizomenon* bloom in Lake Winnisquam near Laconia. In 1966 a toxic bloom was identified in Kezar Lake, North Sutton that caused a mortality of more than 6 tons of fish. Also during the same year a toxic bloom was noted on Skatutakee Lake, Harrisville. Kezar Lake also contained a toxic bloom

of *Aphanizomenon* during the summer of 1967. Toxicity of a unialgal strain isolated from Kezar Lake in 1967 was described by Gentile and Maloney (1969). Intraperitoneal injection of acid extracts from samples of this 1967 toxic bloom, into *Fundulus heteroclitus* (killfish), *Cyprinodon variegatus* (sheepshead minnows), and white mice gave LD_{100}'s of 0.5, 0.5 and 8 mg/kg body weight, respectively. With all toxin extracts tested the mice usually recovered when they survived beyond 15 minutes, having average death times of eight to ten minutes at the MLD. This work indicated no evidence of the SDF, as reported with *M. aeruginosa* NRC-1. Extracts of bacterial contaminants were not toxic to mice. The toxic fraction obtained from *Aphanizomenon* is stated to be similar, if not identical, to saxitoxin (paralytic shellfish toxin), which is produced by the marine dinoflagellate *Gonyaulax catenella* (Jakim and Gentile, 1968).

McLachlan, Hammer and Gorham (1963) described the growth and colony habits of ten unialgal strains of *Aphanizomenon flos-aquae*. They noted that the typical large colony habit or flake form was determined by unidentified factors found in particular soil extracts. O'Flaherty and Phinney (1970) were able to maintain this flake form of a different unialgal strain in a defined medium based on ASM-1 in which the iron was supplied as hydrogen ferric ethylenediamine di-o-hydroxy phenylacetate (EDDHA).

Extensive work with toxic strains of *Anabaena flos-aquae* was initiated by isolations from toxic blooms which occurred in Burton

Lake near Humbolt, Saskatchewan in 1960 and 1961 (Gorham, *et al.*, 1964). Seven out of 14 unialgal strains produced a toxin, termed the Very Fast Death Factor (VFDF), which killed mice in one to five minutes after intraperitoneal injection at MLD's of 160 to 1280 mg/kg body weight. The chemical structure of *A. flos-aquae* toxin has yet to be proven conclusively. The fact that it acted more rapidly than *Microcystis* FDF indicates that it has a lower molecular weight (Gorham, 1965). Later work (Stavric and Gorham, 1966) indicated that *Anabaena* VFDF is a low-molecular weight compound that is water- and ethanol-soluble, insoluble in acetone, ether or chloroform, and is a tertiary amine with a strong absorbance at 229 nm. Gorham (1965) cites work done by T. H. Olson which indicates that VFDF kills waterfowl whereas FDF does not. More recent work has shown the toxin to be an alkaloid with a ring system related to that of tropine (Huber, in press). The fact that the toxin of *Anabaena flos-aquae* can be much more toxic than *Microcystis* and kill in less time implicates it as one of the more serious algal toxin producers known.

B. Occurrence of Toxic Blooms

1. General Distribution and Nature of Toxic Blooms. Poisonings by waterblooms of blue-green algae are almost world-wide in occurrence. Several good reviews of algal poisonings exist with one of the earliest being that by Ingram and Prescott (1954). Schwimmer and Schwimmer (1964, 1967) have provided a listing of all reported cases of algal poisonings and laboratory work with natural and

laboratory-grown cultures. They have also provided a listing of reported animal intoxications caused by algae from a period of about 1878 to 1965. General symptoms of the poisonings, as given in the 1964 review, involve those of the neuro-muscular system including spasms, twitchings, convulsions, weakness, uncoordination and paralysis. Following this there is a lethargy verging into stupor and death. Post-mortem examination shows only congestion of meninges and cerebro-spinal blood vessels. Reviews dealing with the three most implicated genera of toxic blue-greens (*Microcystis*, *Aphanizomenon* and *Anabaena*) are provided by Gorham (1964 a, 1965).

Many of the reports on toxic blue-green blooms have noted the variability of their toxicity as revealed by animal losses and the generally frequent occurrence during one year in a particular water body but not in previous or subsequent years. It is then implied that toxicity of algal blooms is affected by many different conditions present at the time of bloom and before toxic bloom accumulation. The fact that toxicity of blooms varies so much makes them potentially dangerous and also poses problems for investigating the causes. Factors involved in the variable toxicity of blooms in nature have been discussed by Gorham (1965). These include differences in algal and bacterial species and strains, growth conditions, age, accumulation, secretion, destruction or inactivation by adsorption to sediments, dosage and animal susceptibility.

The greatest mortality of animals due to toxic algae occur when blooms accumulate near a shoreline by the prevailing winds. During these times of heavy accumulation, oxygen tension and extracellular products secreted by the lysing algal cells become potentially important factors that could affect toxin variability. Oxygen tensions in blooms in an advanced state of decomposition can fall quite low (one to two parts per million, personal observation). Mackenthun, Herman and Bartsch (1948) have noted low oxygen tensions in a heavy bloom of *Aphanizomenon flos-aquae*. The low oxygen tensions resulting from the bloom were held to be the primary cause for many fish deaths with secondary causes being attributed to toxic substances secreted into the water. Prescott (1948, 1960) implicated hydroxylamine as causing fish deaths when decomposing *Aphanizomenon* was added to tanks containing the fish.

The extracellular products secreted by a variety of algae have been discussed by Lefèvre (1964) and Fogg (1962, 1965). They include enzymes, polypeptides, polysaccharides, amino acids, vitamins, growth factors, antibiotics, steroids, saturated and unsaturated fatty acids, and organic acids, as well as toxic and growth-stimulating factors. Extracellular products of blue-greens which have been described are similar to those of other classes of algae (Gorham, 1965). Fogg (1965) outlines a number of the roles of these extracellular products of algae. They include rapid turnover of fixed carbon, chelation of heavy metals, and serving as precursors for proteins, vitamins or growth factors. The identification and specific

role of most extracellular products from *Anabaena flos-aquae*, other than the toxin, and a heteropolysaccharide reported by Moore and Tischer (1965) from an axenic strain, is not known. Because toxin levels can be so high during bloom accumulation and decomposition, when partial to full anaerobic conditions are observed it was thought necessary to study the possible effects of oxygen tension on toxin production and accumulation in *Anabaena flos-aquae*.

Most toxic algal blooms from Canada have been reported from the provinces of Saskatchewan and Ontario. There have been a number of serious outbreaks in Alberta over the years and O'Donoghue and Wilton (1951) have provided an account of some of those which occurred in the summer of 1950. Because of the problems involved with collection, identification and distance to the toxic algal bloom locations, the only observations which could be made concerned the animals affected and the general nature of the heavy algal blooms. Samples sent to the University of Alberta were too decomposed for species identification but were classified as *Microcystis* sp. These toxic blooms were located on the shores of Duck Lake and Baptiste Lake, which are situated one hundred miles northeast and north of Edmonton, respectively. Other records of toxic blooms from Alberta on file with the Alberta Department of Agriculture Veterinary Services Division, describe blooms from lakes near Coronation in May, 1957, and Vilna in September, 1968. The Vilna bloom (Plate 1) consisted predominantly of *Anabaena flos-aquae* as identified by Dr. L. L. Kennedy, Department of Botany,

PLATE 1A. Dyzak Lake on Nick Dyzak property, view from S.E. corner of lake, decomposing toxic *A. flos-aquae* bloom at lower right. Photograph by P. W. Frobb, M.D., Vilna, Alberta. Sept. 17, 1968.

PLATE 1B. Closeup of decomposing toxic *A. flos-aquae* bloom, S.E. corner of lake. Photograph by P. W. Frobb, M.D. Sept. 17, 1968.

University of Alberta. The MLD (IP, mice) of the bloom as measured on a partially decomposed sample sent to Prof. P. R. Gorham in Ottawa, Ontario was 4 mg dry weight per kg body weight. This was approximately five to ten times greater than the most toxic laboratory-grown culture of *Anabaena flos-aquae* known to that date. No cases of algae poisoning have been reported from this lake in the succeeding three years, however.

2. Occurrence of Toxic *Anabaena flos-aquae* Blooms and Some Case Histories. Reports of toxic *A. flos-aquae* waterblooms have come mainly from North America. Schwimmer and Schwimmer (1967) indicate that toxic *A. flos-aquae* blooms have been reported for the states of Iowa, Colorado and Minnesota and the provinces of Manitoba, Ontario and Saskatchewan. Alberta, as previously mentioned, also has toxic *A. flos-aquae* blooms on record. Toxic *A. flos-aquae* blooms have been reported from Finland and Sweden as well.

One of the best documented case histories of a toxic *A. flos-aquae* bloom is one which occurred at Storm Lake, Buena Vista County, Iowa (Rose, 1953). In the fall of 1952, after a long dry summer, heavy quantities of *Anabaena* with lesser amounts of *Aphanizomenon* and *Microcystis* filled all of the lee shores and bays of this relatively shallow lake. Early in October, 1952, an estimated 2,000 Franklin's gulls died and microscopic examination of lake water on October 13 showed almost complete dominance by

A. flos-aquae. The plants at this time were green with no evidence of decomposition. Again on October 29, a heavy loss of Franklin's gulls occurred with approximately 5,000 dead or dying, on the calm lake surface. On November 16 the lake was very calm and another loss occurred, mainly of Mallard ducks. Animals that died during these times besides waterfowl included fox, squirrels, muskrats, dogs, cats, hogs, skunks and mink. In addition, amounts of the algae fed to chickens, guinea pigs, rabbits and mice in the laboratory resulted in death in all cases. Heavy losses were interrupted during these sequences of toxic blooms by wind action which broke up the surface bloom. It is also possible that other factors present caused a temporary decline in toxin production or accumulation. No toxic blooms were recorded for the following year and algal blooms in general were kept to low amounts by treatment of the lake with copper sulfate.

Samples of toxic *A. flos-aquae* from Burton Lake near Humbolt, Saskatchewan which were collected in 1960 and 1961 also illustrate the variable toxicity of blooms over a period of time (Gorham, *et al.*, 1964). Samples collected on three dates during 1960; August 31, September 14 and September 24, varied both in toxicity of the algal and water fractions. The September 14 sample had an MLD of 320 for the algal fraction and an MLD of 40 for the water fraction while the September 24 sample had an MLD of 160 for the algal fraction and an MLD of 80 for the water fraction. The

August 31 sample had a combined MLD of algae plus water of 160 but no individual MLD's were reported. It is thus evident that factors were present which caused varying amounts of toxin to accumulate in the cells or appear in the surrounding medium over a 10-day period.

C. Laboratory Observations on Culture and Toxicity of *Anabaena flos-aquae*

1. Culture Medium and Growth. An optimal mineral medium for growth of unialgal colony isolates of *A. flos-aquae* in shake flasks at 22°C under continuous illumination of 1500 lux from cool white fluorescent lamps has been developed by Gorham, *et al.* (1964). The medium was termed ASM-1 since it is a modification of ASM (Artificial Seawater McLachlan) which was developed for optimal growth of *Microcystis aeruginosa* NRC-1 (McLachlan and Gorham, 1961). Growth of *A. flos-aquae* and, indirectly, toxin production which depends on it are affected by the composition of the major and minor elements in the mineral medium. While some work has been done with *A. flos-aquae* on specific effects of different elements on growth and morphology (Gorham, *et al.*, 1964; and Bostwick, Brown and Tischer, 1968), none has been done on their effects on toxicity. Gorham, *et al.* (1964) noted compensating effects of iron and manganese on filament length and coiling of the unialgal colony isolate *A. flos-aquae* NRC-44. Bostwick, *et al.* (1968) studied the

essentiality and interactions of potassium and sodium for an axenic culture of *A. flos-aquae* A-37.

From general reviews on algal nutrition such as that by Krauss (1958) and the review of microinorganic requirements by Eyster (1964), certain ideas can be gained about relationships between growth and mineral nutrition of *A. flos-aquae*.

There existed a lack of information on direct effects of mineral nutrition on VFDF synthesis or on indirect effects on toxicity through growth and development of *A. flos-aquae*. Due to this it was important to investigate the effects of a range of concentrations of the essential elements on toxicity of cultures at different stages of their growth.

2. Colony Isolates and Toxin Variability. Cultures of *A. flos-aquae* grown in the laboratory have also been found to vary greatly in toxicity. From two toxic blooms which occurred in Burton Lake, Saskatchewan in 1960 and 1961, 14 unialgal colony isolates of *A. flos-aquae* were successfully grown in ASM-1 medium and tested for toxicity. Bioassay results indicated that eight were nontoxic and six were toxic. The toxicity of one strain (NRC-44) varied from zero to a trace in tests on three successive subcultures, while that of another strain (NRC-36) varied ten-fold between two successive subcultures grown under the same conditions (Gorham, *et al.*, 1964). Later work with unialgal clones derived by single filament isolations from two of the toxic strains mentioned

above, NRC-36 and NRC-44, gave varying toxicities after growth for 21 days under the same physical conditions. Four of the clones from NRC-36 gave toxicities ranging from 10 to 45 MU*/liter and eight clones from NRC-44 gave toxicities ranging from 20 to 125 MU/liter (Gorham, personal communication).

3. Influence of Bacteria on Growth and Extracellular Products of Blue-green Algae. Interactions between bacteria and blue-green algae have been recognized for some time but only recently have there been critical studies on the isolation and identification of lytic bacteria for some blue-green algae (Daft and Stewart, 1971; Shilo, 1971; and Stewart and Brown, 1971). Daft and Stewart isolated lytic bacteria from sewage works and from freshwater lakes of the Dundee region of Scotland. *Anabaena flos-aquae* A-37 (Tischer's strain) and the Windermere strain of *A. flos-aquae* were both susceptible to the four lytic bacteria isolated. It was also revealed that intact bacteria were necessary for lysis which occurs from two to ten hours after contact. The work of Shilo indicates that direct contact is necessary and that the polar end of the bacteria and the algal cell must remain connected for 10 to 20 minutes for lysis to occur. In all cases the bacteria have been placed in the order Myxobacteriales.

In the presence of bacteria there is evidence to indicate that much of the extracellular substances of algae are used up

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*MU = Mouse Unit, and is defined as the minimum dosage to kill a 25-gram mouse.

(Fitzgerald, 1964). For the current investigation with toxin production and accumulation in *A. flos-aquae* this latter information has great importance. It was thought that bacteria might degrade some or all of the exogenous toxin of a toxic algal bloom, which ranges from 50% with healthy filaments to 100% with lysed filaments.

D. Purification Methods for Obtaining an Axenic Algae Culture, Especially Planktonic Blue-greens

1. General. The bacteria and algae should be separated in order to effectively study algae physiology or interactions between bacteria and algae. This is particularly hard to do with planktonic blue-green algae as most methods used to eliminate the bacteria affect the algae as well. Axenic cultures of brown, red and green algae can in most cases be obtained quite easily by the use of antibiotics and some micro-manipulation. For example, antibiotics can be used to surface-sterilize portions of the brown or red algae; the sorus in the case of some brown algae and the tips of the alga thread with some red algae (Druehl and Hsiao, 1969; and Fries, 1963). Provasoli, *et al.* (1951) and Droop (1967) have provided procedures for antibiotic purification of green algae as well as other algal orders.

2. Axenic Cultures of Blue-green Algae. Methods for obtaining pure cultures of algae have been investigated for many

years. Information on pure culture technique and references to the earlier literature have been reviewed by Pringsheim (1946). Concentrations of antibiotics needed to eliminate bacteria from blue-green cultures are usually in excess of that tolerated by the algae. Because of this, most successful attempts at obtaining axenic blue-green cultures use either radiation or physical separation. One of the first reported successful attempts at purification of planktonic blue-green algae was by Gerloff, Fitzgerald and Skoog (1950). Their axenic cultures were obtained by irradiation for 20 to 30 minutes with ultraviolet light at 2750 A^{o} from a quartz-jacketed mercury vapor lamp. Samples were removed from the irradiation chamber at five minute intervals and a large number of dilution cultures were made. Some algal cells which were more resistant to ultraviolet than the bacteria were diluted to a point where no bacteria were present. Kraus (1966) and Lange (1970) report successful attempts to obtain axenic blue-green cultures by use of gamma radiation from a ^{60}Co source. Lange exposed tubes containing the algae in culture to varying radiation doses in the range of 0.14×10^6 rad to 4.5×10^6 rad. Assumed axenic cultures were selected from those that had received the highest radiation doses. These were the cultures that appeared to be dead for about 1 ½ months and started growing within 2 ½ months. In nearly all cases where antibiotics or radiation were used to kill bacteria, erratic and unrepeatable results have been obtained. In

the case of gamma radiation Morton and Derse (1968) report dosages of about 100 to 150 × 10^3 rads as sufficient to control blue-green algae blooms. In order to purify some blue-green algae Lange used dosages in excess of those levels required to control algae blooms. These high radiation conditions account for the low survival rates of exposed algae and indicate that the method involves very marginal conditions for the blue-green algae viability. Another method for obtaining axenic cultures has not received much attention but has possibilities. This method is designed to exploit both the electrophoretic mobility of some ciliated protozoa and flagellated algae and the greater vulnerability of bacteria over protozoa and algae to the lethal effects of an electric field (Coler and Gunner, 1969). Using this electrophoretic mobility technique *Anabaena* as well as other algae and protozoa were obtained in axenic culture.

Physical separation of algae and bacteria, while it maintains some degree of the natural conditions for the organisms, particularly the algae, is a method which is rather specific and must be altered to suit the alga and bacteria present. Basically the method involves separation in or on an agar plate containing suitable growth factors for the algae and bacteria, under which circumstances the alga is separated as an individual filament or cell. This technique was described by Tischer (1965) who obtained *Anabaena, Polycystis (Microcystis)* and *Oscillatoria* in axenic culture. A modification of the above technique was employed by

Stanier, *et al.* (1971) to obtain members of the Chroococcales in axenic culture. They streaked algal colonies on agar plates which contained modified G-11 medium of Hughes, Gorham and Zehnder (1958) (termed BG-11), allowed the algae to grow and isolated colonies of algae located well away from the bacteria. This was repeated until all contaminating bacteria were eliminated. This method required up to six months for some purifications and it was necessary for the algae to grow on the agar surface. *Anabaena flos-aquae* NRC-44 does not grow well on an agar surface but does grow well, under the proper conditions, when embedded in agar. Kim and Gorham (Gorham, personal communication) after trying procedures such as antibiotics, disinfectants, bacteriostatic agents, ultraviolet radiation, infinite dilution and single cell isolation without success obtained NRC-44-1 as an axenic culture using a technique similar to Tischer's. The method consisted of increasing the ratio of algae to bacteria by repeated washing of an aliquot of culture in ASM-1, then adding the washed algae to almost-solidified washed agar plus vitamins and peptones (ASM-2). Upon growth of algae and bacteria, isolated algal filaments that appeared to be bacteria-free were separated by coring the agar and transferring the cores to ASM-1 medium in screw-top test tubes. The filaments were grown and tested for purity by phase contrast microscopy and 10 to 20 different bacteriological media. This method was successfully used to purify various species of *Nostoc* and *Anabaena* by Kantz and Bold (1969).

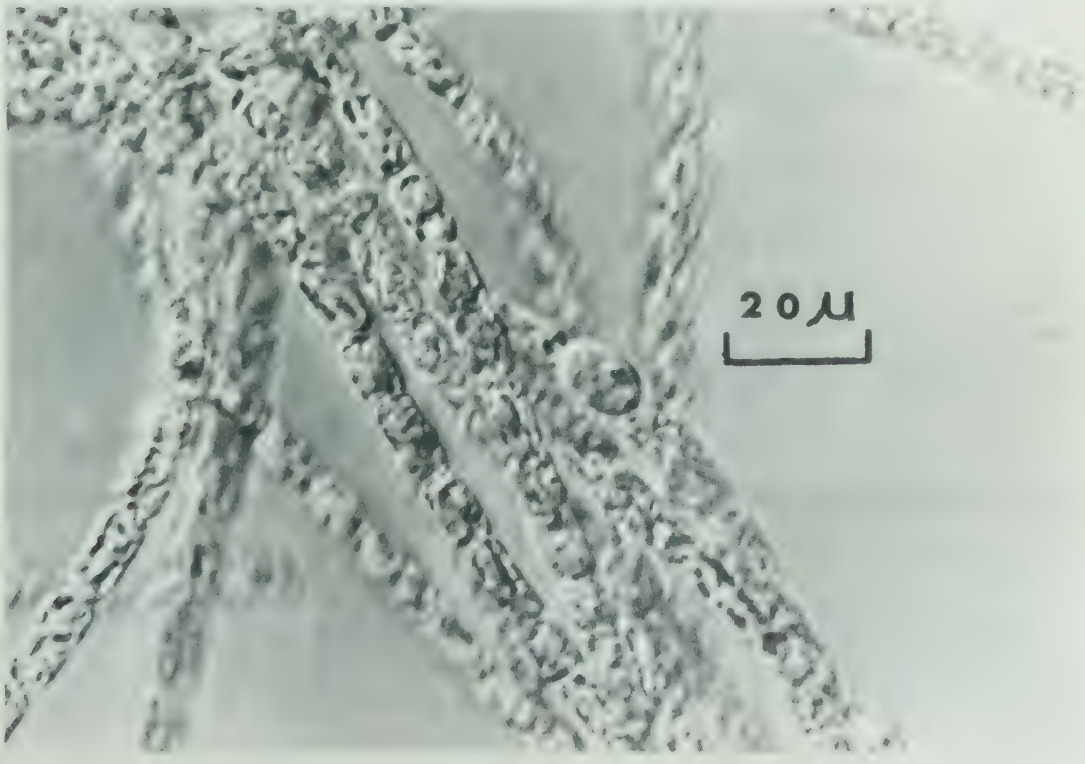
MATERIALS AND METHODS

A. Culture Conditions

Anabaena flos-aquae NRC-44-1 used in the present research is a clonal isolate of NRC-44 from the Burton Lake, Saskatchewan bloom of 1961 (Gorham, *et al.*, 1964) (Plate 2). It is the isolate purified by the method of Kim and Gorham but which later became contaminated by several different species of bacteria. Cultures for the experiments conducted were grown in 250-ml Delong flasks containing approximately 50 ml of ASM-1-Tricine (ASM-1-TR) (Appendix I), then transferred every 10 to 14 days into fresh ASM-1-TR medium using a two to three per cent inoculum. All cultures were grown at 22 ± 1°C under continuous shaking and illumination of 4000 to 5000 lux, as measured at the level of the culture with a Gossen photocell and meter, from overhead cool white fluorescent lamps. These physical conditions of growth have been found by Peary and Gorham (Gorham, personal communication) to be close to the optimal conditions for growth and toxin production. Aerating axenic NRC-44-1 under these conditions for 21 days, they obtained 1100 MU/liter while 15000 lux gave 500 MU/liter. Lowering the temperature to 15°C and 5000 lux gave 300 MU/liter while only a trace of toxicity was detected at 15000 lux. In all cases toxin production was governed by growth.

For the current investigation two types of rotary shakers were used. One was a small unit operated at 80 to 100 rpm which was

PLATE 2. *Anabaena flos-aquae* NRC-44-1
(Nomarski interference contrast)



built by University Technical Services, on which the Delong flasks were shaken. The other type was a large New Brunswick, model G-10, operated at 60 to 80 rpm, on which the specially designed 3-liter culture flasks equipped for aeration (Plate 3) were shaken. These had cotton-stoppered inlet and outlet ports. In experiments comparing growth and toxicity of nonaxenic with bacteria-specific and axenic cultures, sterilized thistle tubes filled with glass wool were attached to both inlet and outlet air lines for additional control of air-borne contamination.

B. Measurement of Growth

Algal growth was measured as biomass production based on optical density (OD) readings at 750 nm at time intervals indicated with the individual experiments. Optical density readings at 750 nm were taken using three ml of suspension in a cuvette of one centimeter light path on a Beckman DB-G grating spectrophotometer. A standard curve was obtained by concentrating a 7- to 10-day old aerated culture by Millipore filtration and suspending the cells in deionized water to give a range of OD values. Fifteen milliliters of each cell suspension was then vacuum-oven dried in a tared 30-ml beaker and weighed, taking the average of four replicates. Using this method, linear values of OD versus dry weight up to about 0.50 OD units were obtained (Figure 1). Figure one is thus the linear regression of OD 750 nm on dry weight over the given OD range and is significant at the 99% level (Snedecor and Cochran, 1967). Above

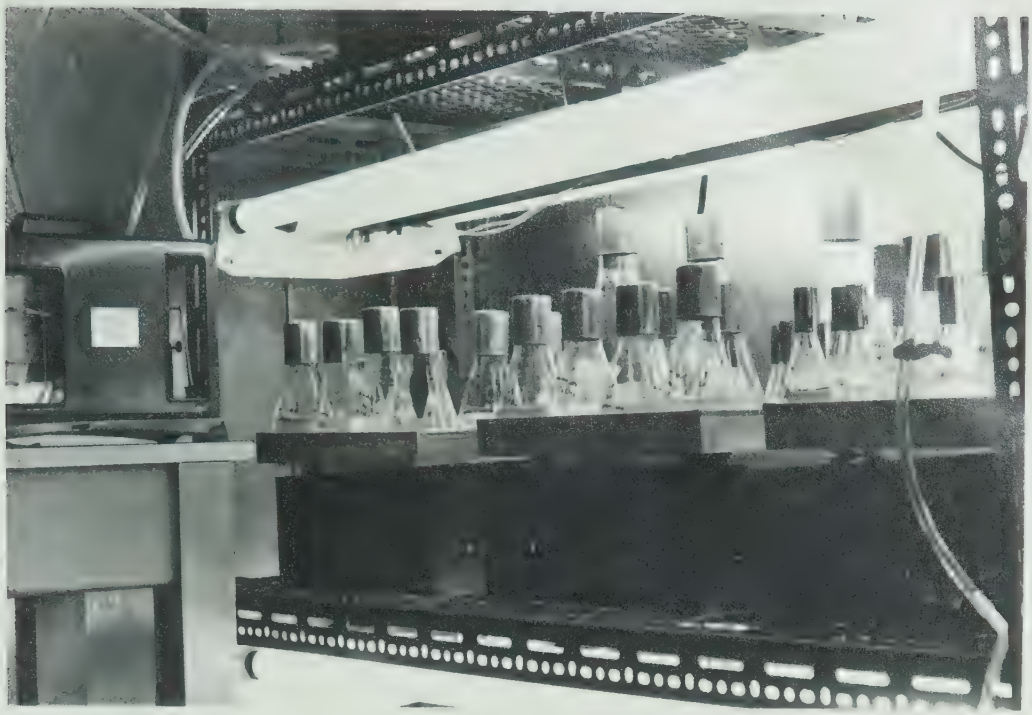


PLATE 3. Top. Small rotary shakers for Delong flasks.
Bottom. New Brunswick, model G-10, shaker and flask system.

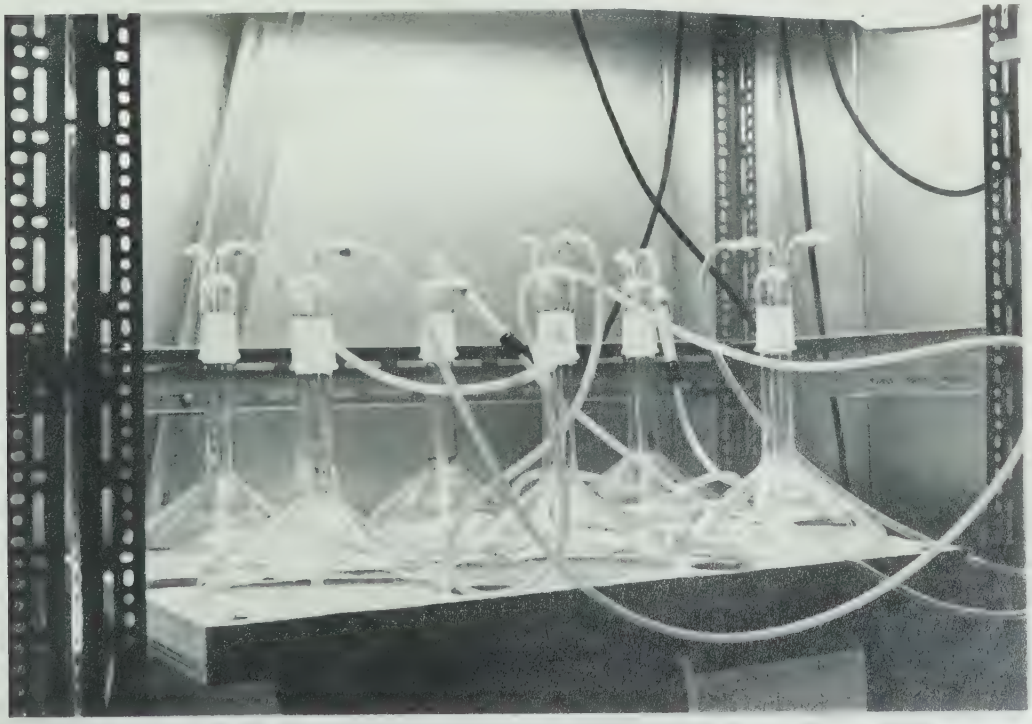
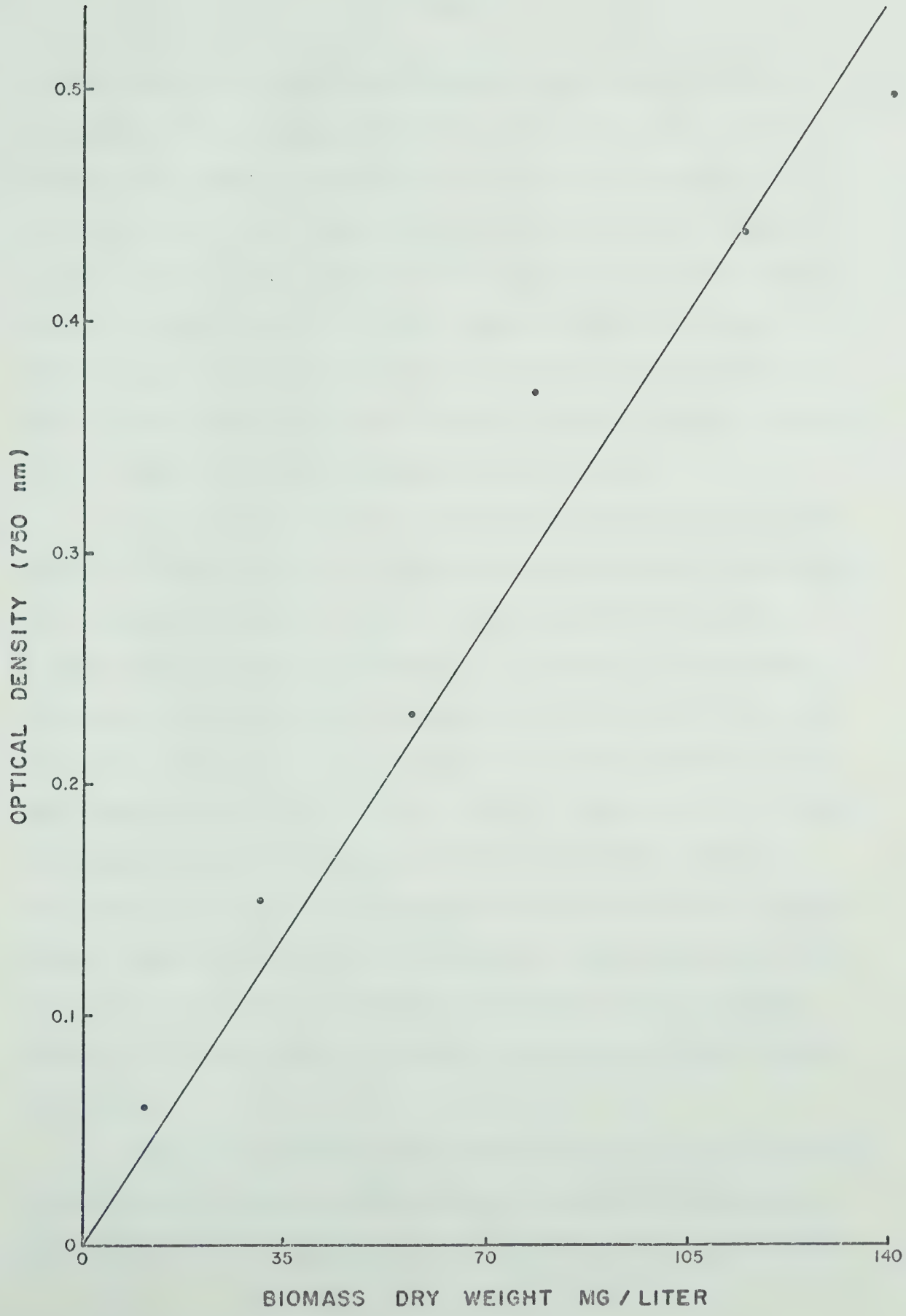


FIGURE 1

Regression of OD (750 nm) on biomass dry weight per liter, over the range from 0 to 0.5 OD units.


OPTICAL DENSITY (750 nm)
0.5
0.4
0.3
0.2
0.1
0
0
35
70
105
140
BIOMASS DRY WEIGHT MG / LITER

0.5 OD units the straight line relationship does not exist and a curvilinear line is found. For this region of the graph, small differences in OD correspond to relatively large differences in dry weight per liter. Kumar and Gorham (Gorham, personal communication) found a similar range of linearity of OD versus dry weight using OD values at 670 nm. Optical density readings were made at 750 nm because with this alga there is minimal absorbance from pigments at this wavelength and OD readings are then primarily a measure of the number of filaments present per unit volume.

The foregoing method of growth determination measures the amount of biomass present. A standard growth curve was also obtained by diluting a concentrated algal culture into ASM-1-TR, drying in a vacuum oven, weighing and subtracting the amount of salts present as determined from an equivalent amount of dried ASM-1-TR. This salt correction factor was close (305 mg/liter) to that determined from the formula weights of the salts in ASM-1-TR (Appendix III). Ashing of samples in a muffle furnace at 550^{o}C for two hours was also utilized to check on salt concentration at a given OD value. Results from a sample with an OD of approximately 0.5 gave 62% salt and 38% biomass. This biomass concentration corresponds with the biomass content of a sample with an identical OD value dried by the vacuum oven method.

Determining total weight (biomass plus salts) from a freeze-dried aliquot is also possible but loss of sample by adherence to the surface of the flask and uncontrolled losses under vacuum make

this method generally unsatisfactory. Although every effort was made to be as quantitative as possible, the error after freeze-drying and weighing a known amount of ASM-1-TR was about 20%.

C. Bioassay Techniques and Criteria

In the present work the mouse bioassay as employed in previous work (Hughes, *et al.*, 1958) was used for toxicity determinations since no satisfactory chemical method was available. A 64 mg freeze-dried sample of culture material was diluted with two milliliters of deionized water. Intraperitoneal injection of 0.1 ml/10 g body weight of this sample gave a 320 mg/kg body weight dosage. Further dilutions were made depending on what toxic level was known or suspected, using a standard dilution series of 320, 160, 80, 40, *etc.*, mg/kg body weight. For any one dosage a minimum of two weighed mice were injected using a 1 cc tuberculin syringe. Mice used in the tests were 15 to 20 g (occasionally 20 to 24 g) white males of the Alberta inbred "ALAS" strain obtained from the University of Alberta Bioscience Animal Services. Male mice were used since earlier workers used them and because female mice of the same weight exhibit slightly more resistance to the VFDF toxin. If all mice injected at a given dosage died, the next lower dosage level was tried until the minimum lethal dosage (MLD) was found. The result was considered significant if the two mice injected died. If one of the two mice died, two more were injected and the result was

considered significant if three out of four died. Mice weighing greater than 24 g were not used because 24 g is a critical minimal weight before the onset of an increased resistance to VFDF. The almost all-or-none nature of the response of mice to intraperitoneal injection with VFDF made it possible to use the MLD rather than the LD_{50} (50% lethal dosage) as the measure of toxicity. Likewise this made it possible to use duplicate rather than a larger number of mice per dose and still obtain results that were of practical significance. The time which elapsed between injection and death was also an indication of potency. Dosages producing death times of five to seven minutes were assumed to be close to the MLD while those producing death times of two to three minutes usually meant excessive toxin. Results from any injection in which leakage of sample or bleeding were observed were discarded and the test repeated. MLD differences were not regarded as significant unless they varied by one or more dilution steps from one another. This is because replicate variability is no better than plus or minus one-half step.

Toxicity was also expressed in terms of Mouse Units (MU). Data were then expressed either as MU per liter or MU per mg dry weight plus and minus salts. This was done by the following procedure:

1) There are 40 MU/kg by definition

2) MLD (mg/kg) ÷ 40 (MU/kg) = x (mg/MU)

3) dry weight of sample (mg/liter) ÷ x (mg/MU) =
y (MU/liter)
(Dry weight per liter was expressed both as biomass plus salts and as biomass only)

4) y (MU/liter) ÷ mg biomass/liter = z (MU/mg biomass)

Total dry weight determines the MU present in a liter of culture but since all MU are related to biomass it is better to represent toxicity on a unit biomass method. It was also noted that with increasing amounts of mineral elements MLD can increase. This is particularly true for components that are a major percentage of the salts in ASM-1-TR, *e.g.* NO_3 in Appendix V, Table A. Increased salt content decreases the amount of toxin present in a standard sample and MLD's are ultimately affected. Because of this MU/mg biomass is a better measure of toxicity.

D. Repurification of NRC-44-1

The method used to repurify NRC-44-1 was similar to that used by Kim and Gorham (Gorham, personal communication) but employed several important changes. These changes are outlined in a section under "Experimental Conditions and Results." The methods used to test for purity of a culture were also similar to those of Kim and Gorham and involved positive phase contrast illumination microscopy and selective bacteriological test media. For microscopic examination, a slide mount of a culture 14- to 21-days old was examined closely for bacterial contaminants. Low-level con-

taminants could be detected this way even when not discovered by the selective bacteriological media.

The selective media used to test for purity were chosen to grow the widest range of organisms which might exist in the cultures. The test media were incubated at 22, 28 and 37°C and checked periodically for growth over a time span of one to three weeks. The media used are listed below in order of preference. The compositions of all media marked with an asterisk are given in Appendix IV. Test media marked with this symbol † were used by Kim and Gorham (Gorham, personal communication) in the original purification procedure for NRC-44-1.

*Gelatin - Vitamin - Peptone Medium

*Sodium Caseinate Agar†

Plate Count Agar (Difco)

Sodium Carboxymethylcellulose (3%)

*Lochhead's Soil Extract Semi-solid Medium†

Ascorbic Acid Broth in ASM-1-TR†

ASM-2 plus 2% Soil Extract†

Potato Dextrose Agar (Difco)†

Maize Agar†

*Casitone Agar

Brewer Thioglycollate Medium (Difco)†

Tryptone Broth (Difco)

Azotobacter Medium†

EXPERIMENTAL CONDITIONS AND RESULTS

A. Oxygen Tension

Seven- to ten-day old cultures grown on large shaker flasks were concentrated to an OD value of approximately 1.0 by low speed centrifugation, accelerating to 650 *g* and immediately decelerating without braking. Centrifugation was at low speed because Kim and Gorham (Gorham, personal communication) had observed that 1000 *g* is sufficient to cause pronounced growth inhibition. One hundred and fifty milliliters of this culture adjusted to an OD of 1.0 was placed in 200-ml Erlenmyer flasks which were rubber-stoppered, connected and aerated in series with compressed gas mixtures of 0, 2 and 21% oxygen at a flow rate of 2 liters per hour. Samples were then removed at times of 0, 12, 24 and 32 hours and freeze-dried for a mouse bioassay. The principal treatments were carried out in the dark but one part was performed under 12 hour dark 12 hour light at 4000 to 5000 lux. For fully anaerobic experiments (zero per cent oxygen) N_2 gas was deoxygenated by bubbling through alkaline potassium pyrogallate before use. The partial anaerobic condition was represented by the two per cent O_2 concentration. Oxygen tensions were measured with a Beckman, model 1008, O_2 Field Analyser with a platinum electrode. This instrument was calibrated by: 1) passing deoxygenated N_2 through water and allowing water-saturated gas to

pass over the electrode to give the 0% reading; 2) passing pure O_2 through water and over the electrode to give the 100% reading and 3) passing building compressed air through water and over the electrode to give the 21% reading. Correction of the O_2 content of the compressed air for barometric pressure was small enough to be neglected.

Centrifugation, as described, resulted in some loss of gas vacuoles (also termed pseudovacuoles) which appeared to be restored to normal by at least 12 hours. Centrifugation to 650 *g* also had little effect on toxicity as the MLD's of a test culture, before and after such treatment were 80 and 60, respectively.

Preliminary experiments had shown that complete lysis of the culture occurred between 32 and 40 hours under anaerobic growing conditions so that 32 hours was chosen as the maximum test period. Because OD values were greater than 0.5 the dry weight determinations were made by drying samples in the vacuum oven.

From Table 1, neither biomass dry weight nor specific toxicity (MU/mg biomass) varied greatly with oxygen tension over the test times or test exposures. In combination with the 24 hour diurnal light treatment 21% O_2 tended to increase toxicity above the control value while 0% O_2 had the opposite effect. The difference between the two extremes appeared to be significant since there was an MLD variance of a whole dilution step. No tendency for change in toxicity was observed with 2% O_2.

TABLE 1

The effects of oxygen tension on growth and toxicity of a ten-day old culture of nonaxenic *Anabaena flos-aquae* NRC-44-1 treated in the dark and in 12 hour dark, 12 hour light.

Treatment		MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	MU/mg Biomass
Time, Hours	O_2, %				
Control		240	683	378	.304
	DARK				
12	21	240	687	382	.301
12	2	160	705	400	.437
12	0	240	686	381	.301
24	21	160	733	428	.432
24	2	160	697	392	.446
24	0	240	782	477	.272
32	21	160	697	392	.446
32	2	240	609	304	.328
32	0	240	707	402	.298
	DIURNAL LIGHT				
24	21	160	744	439	.421
24	2	240	783	478	.271
24	0	320	701	396	.214

B. Mineral Nutrition

Concentrations of major and minor elements were varied in most cases from 0 to 10 times the amount present in ASM-1-TR culture medium. Five hundred milliliters of culture were grown under nearly optimum conditions using one-liter Delong flasks and 1% v/v nonaxenic inoculum of 0.2 to 0.3 OD. In most cases at time periods of 10, 15 and 20 days, 250-ml samples were taken, OD and pH values recorded, and the appearance of the cultures noted. The samples were then freeze-dried and weighed aliquots were taken for a bioassay of toxicity. The cultures were continuously shaken but were not aerated because facilities were not available for the number of cultures involved. One aerated time series involving the group of all minor elements was run simultaneously with a non-aerated series to test toxicity differences. These mineral nutrition tests were all carried out with nonaxenic NRC-44-1. An axenic clone was not obtained in time to repeat the complete series as originally intended. To partly compensate for this a bacteria-specific clone was used to test possible differences in toxicity with NO_3, Mn, Zn and Fe.

The maximum concentration for each element or group of elements was expected to reach or exceed the 50% toxic level as found by Kim and Gorham using ASM medium (Gorham, personal communication). This level of growth toxicity was not generally reached, however, using the Tricine buffered ASM-1-TR as the basal

medium. "Tricine" (N-methyl tris hydroxymethyl glycine) (Good, *et al.*, 1966) had been used earlier by Kumar and Gorham (Gorham, personal communication) and was found to help growth and reduce lysis of the cultures. Tricine was also less toxic to the cultures than "Tris" buffer which had been used in earlier work (McLachlan and Gorham, 1961).

Because of the large amount of data involved, the complete results of the many tests conducted on mineral nutrition are presented in Appendix V. In general, treatments which stimulated or inhibited growth resulted in a corresponding increase or decrease in toxicity of the cultures as measured by MU/liter. The effects of the individual elements or groups of elements upon growth and specific toxicity (MU/mg biomass being the measure of direct treatment effects upon toxin production) have been summarized and are presented in Tables 2 to 7.

Effects on biomass production by individual major and minor elements or groups of elements are given in Tables 2, 4 and 6. Table 2 indicates no specific effects on biomass with increasing concentrations of the major element components, other than the stimulation of growth by doubling of phosphate concentration. Inhibition of biomass production was noted when sulfate, magnesium or calcium were omitted. Other specific element stimulations or inhibitions might have been seen if tests had been run for more than one culture generation. Specific toxicity for the highest nitrate concentration was not determined because the extra salt increased the MLD beyond the range of the standard bioassay. Table 3 shows corresponding specific toxicity values for the major elements. There was no

TABLE 2

BIOMASS WITH VARYING AMOUNTS OF MAJOR ELEMENTS (ASM-I-TR) FOR 20-DAY OLD CULTURES (N* = 15 DAYS OLD) (MG/LITER)

ELEMENT	MINUS	CONTROL	2X	3X	4X	5X	6X	7X	8X	9X	10X
N*	20	24		24	21		18				
P	26	27	44								
K	29	36				38					34
S	10	29			25			31			
Mg	9	20	20	25							
Ca	18	29		29							

TABLE 3

SPECIFIC TOXICITY, MU / MG OF BIOMASS, FOR MAJOR ELEMENTS (ASM-1-TR), 20-DAY OLD CULTURES (N* = 15 DAYS OLD)

NT = NONTOXIC

ELEMENT	MINUS	CONTROL	2X	3X	4X	5X	6X	7X	8X	9X	10X
N*	1.0	2.3		2.3	1.7		--MLD BEYOND RANGE OF BIOASSAY				
P	1.5	1.6	1.0								
K	1.0	0.8				0.9					1.1
S	2.0	1.0			1.0			1.0			
Mg	2.0	1.4	1.5	1.8							
Ca	1.4	1.4		1.6							

specific stimulation of toxicity over the concentration ranges tested. Some stimulation was apparent with omission of sulfur and magnesium but this corresponded to a decline of growth and concentration of toxin in a smaller biomass.

Table 4 presents effects of minor element variation on biomass production for 15-day old cultures. Some inhibition of growth was noted for varying amounts of the all minor elements group, Mn-Zn in combination, Zn, and chelator as EDTA. The inhibition of growth by all minors can be traced to the zinc component. Together manganese and zinc inhibited growth at concentrations greater than about five times control. Separated it is seen that zinc was the toxic component and inhibition of growth occurred at about six times the control concentration. This toxic effect on biomass production by manganese and zinc can be overcome by increasing the chelator (Appendix V, Table C). Since EDTA is a general chelator it forms a complex with the manganese and zinc so that the amounts of free ions available to the algae are reduced below the toxic level (Wallace, 1962). Walker (1954) observed similar results with *Chlorella* when he noted that addition of EDTA to the nutrient solution increased the need for zinc and manganese but not iron. Inhibition of biomass produced was noted in the absence of chelator while increases in chelator, up to eight times the amount in ASM-1-TR, did not greatly affect growth. Table 5 presents the results that varying the minor element concentration has on specific toxicity. Pronounced inhibition of biomass pro-

TABLE 4

BIOMASS WITH VARYING AMOUNTS OF MINOR ELEMENTS (ASM-1-TR) FOR 15-DAY OLD CULTURES (MG/LITER)

ELEMENT	MINUS	CONTROL	2X	3X	4X	5X	6X	7X	8X	9X	10X
Fe	21	27			30		29				25
ALL MINORS	13	22		21			7				2
Mn; Zn	22	28		24			5				2
Mn	19	24	19	19			26				21
Zn	20	24	17	19			12				2
B; Co; Cu	28	42		36			31				38
B	28	27								30X=24	27
EDTA	3	19		21					22		

TABLE 5

SPECIFIC TOXICITY, MU/MG OF BIOMASS, FOR MINOR ELEMENTS (ASM-1-TR), 15-DAY OLD CULTURES

NT = NONTOXIC

ELEMENT	MINUS CONTROL	2X	3X	4X	5X	6X	7X	8X	9X	10X
Fe	1.3 2.0			0.9		1.4				1.1
ALL MINORS	1.5 2.5		0.7			NT				NT
Mn; Zn	1.2 1.5		0.8			NT				NT
Mn	2.1 2.3	2.1	2.2			1.6				1.3
Zn	2.1 2.3	2.4	1.4			2.3				NT
B; Co; Cu	1.5 1.4		1.2			1.9				1.6
B	1.5 2.0								30X = 1.3	1.1
EDTA	NT. 2.2		1.3					1.5		

duction caused by an excess of manganese and zinc resulted in non-toxicity, with the exception of six times the concentration of zinc which reduced biomass production but specific toxicity was the same as the control. The inhibition of biomass production and specific toxicity was due mainly to the concentration of zinc. High concentrations of the other minor elements had no effects on biomass production and tended to reduce rather than promote toxicity. The absence of iron or all minor elements reduced biomass production and toxicity to varying degrees. The absence of EDTA greatly reduced biomass production and toxicity, presumably because levels of available zinc, other minor elements and iron were now inhibiting growth.

Tables 6 and 7 present the data for biomass production and specific toxicity for the control levels of mineral elements at time intervals of 10, 15 and 20 days. From Table 6 it can be seen that maximum biomass occurred at 20 days for all elements tested except iron which had optimum biomass at 15 days. Results presented in Table 7 show that specific toxicity was greatest at 10 days except for phosphorus which had equal toxicity for 10 and 15 days. Under the experimental culture conditions for these tests the specific toxicity maximums did not correspond with optimum biomass amounts.

The effects of NO_3, Mn, Zn and Fe were repeated using 15-day old cultures of bacteria-specific NRC-44-1. These cultures were grown under standard conditions in one-liter Delong flasks with 500 ml of culture medium at 1% v/v inoculum. The results for these tests are given in Appendix V, Table D. Biomass production

TABLE 6

GROWTH OF CONTROLS, MG BIOMASS / LITER, WITH TIME (DAYS)

	ELEMENT	10	15	20
MAJOR ELEMENTS	N	29	108	138
	P	18	18	27
	K	18	29	36
	S	14	14	29
	Mg	13	16	20
	Ca	19	23	29
MINOR ELEMENTS	Fe	21	31	24
	ALL MINORS	15	29	39
	Mn	18	14	—

TABLE 7

SPECIFIC TOXICITY, MU / MG OF BIOMASS, FOR CONTROLS WITH TIME (DAYS)

	ELEMENT	10	15	20
MAJOR ELEMENTS	N	3.8	2.5	2.1
	P	2.2	2.2	1.6
	K	2.2	0.7	0.8
	S	2.9	1.4	1.0
	Mg	2.0	1.7	1.4
	Ca	2.1	1.8	1.4
MINOR ELEMENTS	Fe	2.0	1.4	1.4
	ALL MINORS	1.8	1.4	0.7
	Mn	2.2	1.9	–

was affected by high zinc concentrations and by both the absence of and increase in concentrations of Mn relative to control. Fluctuations in biomass were evident with varying concentrations of iron but a general trend was not observed. Because of low biomass production involving the individual tests of 0.032 mM zinc and 0.0 mM manganese, the specific toxicities were also higher than controls.

C. Effect of Aeration on Growth and Toxicity

Aeration or CO_2 tension has a pronounced effect on growth but not on specific toxicity. Results of a comparison between aerated and nonaerated experiments for 15-day old nonaxenic cultures (Table 8) are given in Figure 2. Biomass production was over 10 times greater and MU per liter over 15 times greater with aeration. Specific toxicity was not much greater with aeration, however, because the increase in MU was accompanied by an increased amount of biomass. Similar results were found with the duplicate experiment run with the aerated and nonaerated group composed of all minors (Appendix V, Table C). Here, however, specific toxicity was higher for the nonaerated system.

D. Purification Procedure

The method used to repurify NRC-44-1 (Figure 3) was a modification of the method originally used by Kim and Gorham

FIGURE 2

Polygonal diagram to show differences between growth and toxicity for aerated and nonaerated cultures (data from Table 8 using 15-day old nonaxenic cultures; diagraming method adapted from Steward 1971).

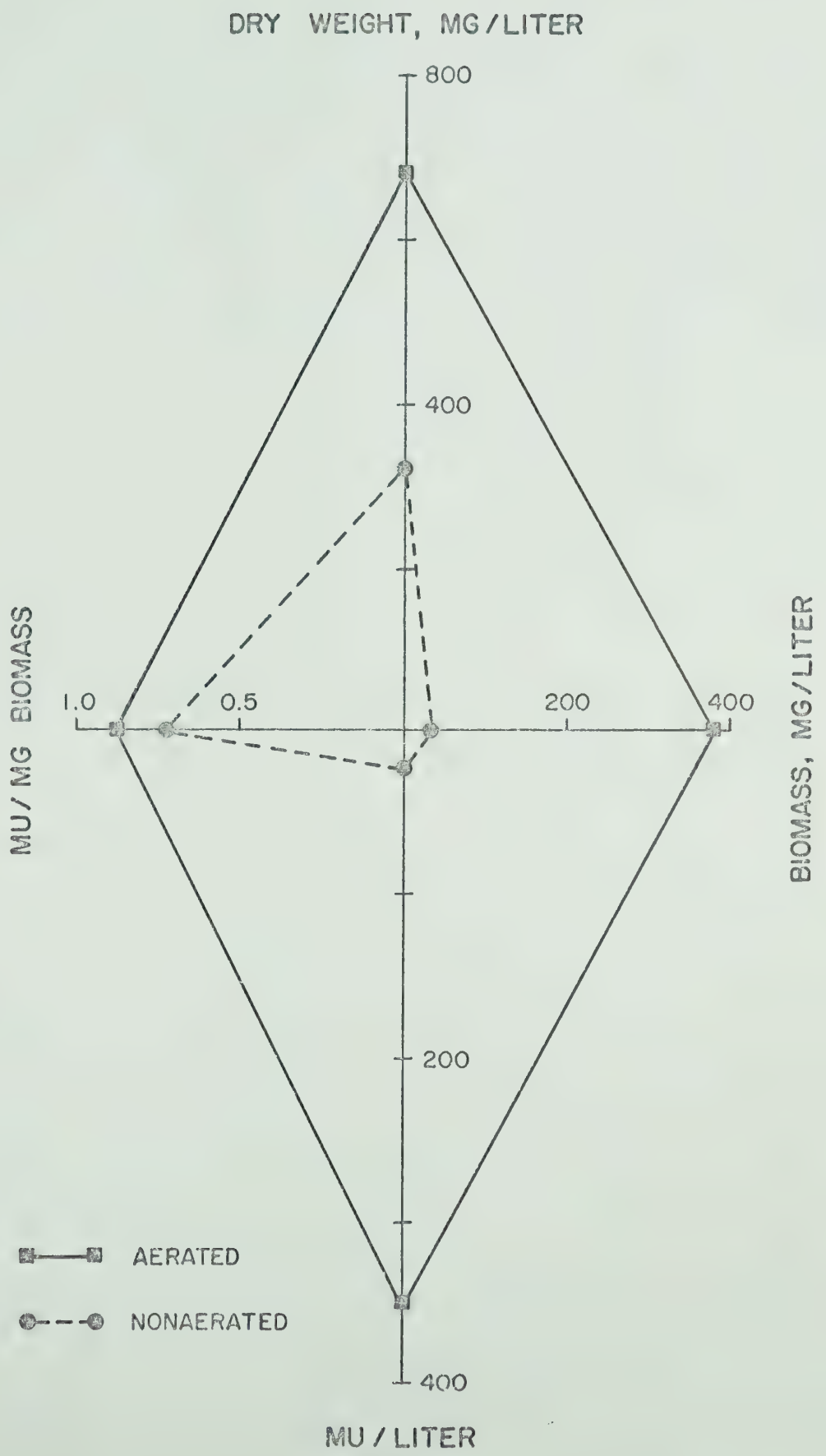

DRY WEIGHT, MG/LITER
800
400
200
400
MU/MG BIOMASS
1.0
0.5
200
400
BIOMASS, MG/LITER
AERATED
NONAERATED
MU/LITER

FIGURE 3

Diagramatic step-wise procedure used to repurify *Anabaena flos-aquae* NRC-44-1.

1- WASH 4X

MILLIPORE PAD

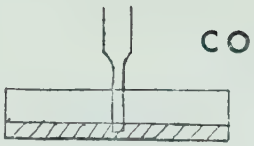
VACUUM LINE

2- POUR; 27°C
ASM-1 S.E. OR ARG.
AGAR (1.0%)
pH 8.5

3- INCUBATE
22°C
2-3 DAYS

4-

5- CORING



6- ASM-2 ; 22° C
4-5 DAYS

CORE

7- (A) PHASE ;
400-1000 X

(B) TEST MEDIA
1-3 WEEKS

8- DARK PHENOL TREAT.
0.1% 4-6 HR

REPEAT 1-7

(Gorham, personal communication) and is described in the following step-wise procedure:

1. Algae Washing Procedure. Five milliliters of 14-day old culture were added to an initial volume of 200 ml sterile ASM-1-TR in a sterile 47-mm Millipore filter apparatus containing the filtering pad but no membrane filter. Suction was applied until only one to two milliliters remained. The pad with adhering algae was transferred to 15 ml of sterile ASM-1-TR, swirled, and the pad discarded. The aseptic washing procedure was then repeated four to five times, changing the sterilized pad each time.

2. Agar Pour-Plates. For initial agar pour-plates, when bacterial contaminants were numerous, a broad spectrum bacteria growth promoter was used. This promoter was soil extract (SE) plus vitamins (Appendix II). One-tenth of a milliliter of the washed algal suspension from the algae washing procedure was stirred, by swirling, into 20 ml of molten ASM-1-TR plus vitamins and SE in 1% washed agar at the critical gel point of about 27^{o}C. Washed agar was prepared by soaking cut-up sections of 2% agar gel in deionized water with daily changes of water for six to eight days. The mixture was quickly poured into two to four petri dishes which were rapidly cooled to room temperature by placing the dishes in the freezing compartment of a domestic refrigerator for 30 to 60 seconds. The plates were inverted and incubated at 22 ± 1^{o}C under continuous illumination of 4000 to 5000 lux. Growth of algae and

bacteria could be detected within 24 hours but incubation was continued for 2 to 3 days. A growing filament, which was free from all visible bacterial colonies, was located with an inverted microscope and removed in its agar core by using a 9" cotton-plugged disposable Pasteur pipette. The agar plug was placed in a test tube with 0.5 to 1 ml of sterile ASM-1-TR and allowed to grow under the same incubation conditions as stated previously. After growth of the algal filament from the agar plug it was transferred in stages to larger amounts of ASM-1-TR. The culture was then checked for bacterial contaminants using the purity tests described in a following section. It was found that one bacterial contaminant still remained which was identified as *Cytophaga* sp. by Prof. F. D. Cook, Department of Soil Science, University of Alberta. This bacteria-specific culture was used for comparing toxin production and accumulation with that of the initial non-axenic culture. A dark phenol treatment which is described in the following section was then used to reduce the population of *Cytophaga* sp. This was followed by a repetition of the washing and agar pour-plate procedures, except that 0.5 mg per 50 ml arginine was substituted for soil extract since it was found that arginine promoted rapid growth of this particular contaminant.

This pour-plate procedure differs from that used by Kim and Gorham in the organic additives to the ASM-1-TR medium as well as in a number of other respects. For the organic additives they used peptones plus vitamins but in repeating this procedure the

peptone batches used were toxic to the alga. Several unsuccessful attempts were made to find concentrations of different peptones which could be tolerated by the alga. The vitamin components were not toxic to the alga but they alone did not promote good bacterial growth. Substituting soil extract for peptones resulted in growth of both alga and bacteria. The concentration of washed agar was reduced from 1.5 to 1% to allow lower critical geling points and thus insure that the detrimental effects of temperature on the algal filaments were reduced. Rapid cooling to room temperature also prevented heat damage to the alga.

3. Dark Phenol Treatment. At the suggestion of Prof. F. D. Cook, an adaption of his dark phenol treatment for selective killing of actively growing bacteria was used to eliminate the *Cytophaga* sp. from the bacteria-specific culture of *Anabaena flos-aquae*. A culture of approximately 14 days was placed in the dark at 22 ± 1°C for about 24 hours. After this period of time, 0.2 to 0.5 mg of sterilized arginine was added to 50 ml of culture and the mixture was returned to the dark (with shaking) for about six hours. Then 0.1% (by volume) phenol was added and the culture was returned to the dark for four to eight hours at which time serial dilutions to about 10^{-6} were made. Phenol treatment may exceed eight hours but 12 to 18 hours seems to be about maximum for retention of algal viability. The washing, plating, coring and purity test procedure was repeated after growth of the serially

diluted cultures, using 0.5 mg per 50 ml arginine instead of SE in the ASM-1-TR pour-plates. This brought up the low-level contaminants that survived the phenol treatment.

4. Purity Tests. Two types of purity tests to check for bacterial contaminants were used; microscopy and selective microbiological test media. Both of these tests are described under the "Material and Methods" section. Two of the microbiological test media, gelatin and sodium carboxymethylcellulose (CMC), used in this current purification procedure have not been used in previous purity tests but were incorporated because they aided growth of the bacterial contaminants in nonaxenic NRC-44-1.

E. Axenic and Bacteria-Specific versus Nonaxenic Cultures

To test possible toxin production and or accumulation differences between axenic, bacteria-specific and nonaxenic cultures, both aerated and nonaerated shaker systems were used. For the aerated system, air was passed through a series of three sterilized thistle tubes filled with glass-wool, before it entered the culture flasks. In later experiments resistance wire was wrapped around the glass exit ports to provide localized heat to prevent condensate formation which might cause bacterial contamination.

The method used to repurify NRC-44-1 resulted in a stepwise series of gradually purer cultures. The bacteria present

after the first washing, plating and coring procedures appeared to be *Cytophaga* sp. as mentioned earlier. Because of the importance of *Cytophaga* in blue-green algae lysis it was necessary to compare toxicity of a culture containing this bacteria with a nonaxenic and an axenic culture. Specific toxicity of both bacteria-specific and axenic cultures were found to be greater than that of the nonaxenic culture (Table 8). The specific toxicity of the bacteria-specific and axenic cultures were not greatly different indicating that the *Cytophaga* sp. present was not having any effect on toxicity. Figure 4 presents schematically the difference in toxin levels for the three cultures under conditions of shaking and aeration. The results show that while total dry weight and biomass weight are similar, specific toxicity varied considerably, being greater for the axenic and bacteria-specific cultures.

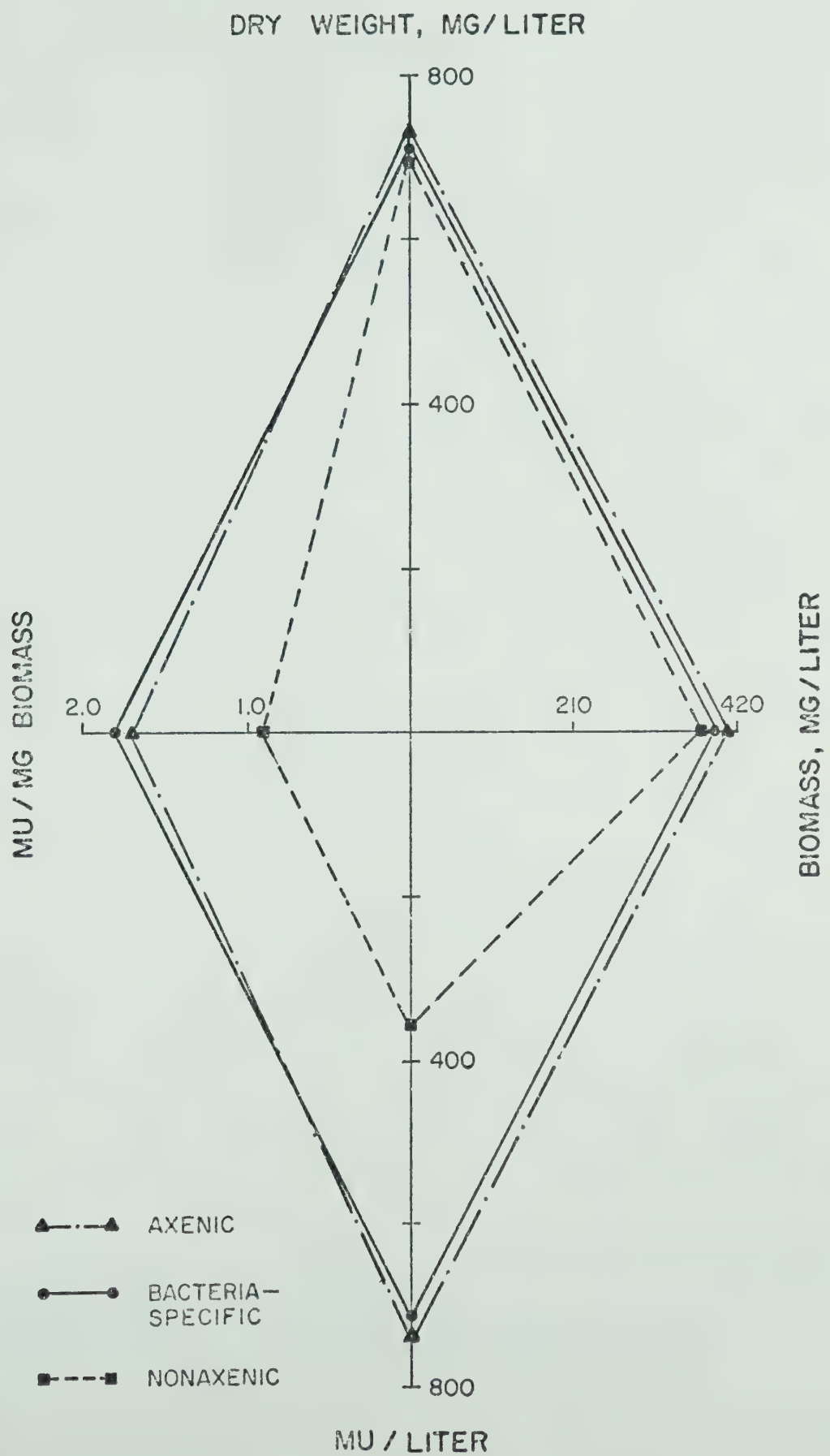
TABLE 8

Anabaena flos-aquae NRC-44-1 -- Growth and toxicity for axenic, bacteria-specific and nonaxenic cultures at 10, 15 and 20 days, both aerated and nonaerated.

	Component	Age, Days	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
Nonaerated	Axenic	10	480	327	22	27	1.2
		15	240	336	31	56	1.8
		20	160	348	43	87	2.0
	Bacteria-Specific	10	480	317	12	26	2.2
		15	240	324	19	54	2.8
		20	240	331	26	55	2.1
	Nonaxenic	10	320	323	18	40	2.2
		15	640	334	29	21	0.7
		20	480	341	36	28	0.8
Aerated	Axenic	10	80	414	109	207	1.9
		15	40	715	410	715	1.7
		20	40	---	---	---	---
	Bacteria-Specific	10	80	411	106	205	1.9
		15	40	705	400	705	1.8
		20	60	---	---	---	---
	Nonaxenic	10	160	395	90	99	1.1
		15	80	690	385	345	0.9
		20	120	---	---	---	---

FIGURE 4

Polygonal diagram to show differences between growth and toxicity for 15-day old aerated cultures of axenic, bacteria-specific and nonaxenic NRC-44-1 (Data from Table 8).


DRY WEIGHT, MG/LITER
800
400
400
800
2.0
1.0
210
420
MU / MG BIOMASS
BIOMASS, MG/LITER
MU / LITER
AXENIC
BACTERIA–SPECIFIC
NONAXENIC

DISCUSSION

The basic goal of this study was to gain a better understanding of some of the factors, in addition to those already known, that cause variable toxicity in *Anabaena flos-aquae* water-blooms. The variable occurrence in intensity and duration of algal poisonings of livestock and wild animals, particularly by *A. flos-aquae* is a problem which cannot be satisfactorily explained by only the fact that there are toxic and nontoxic strains. The variable intensity and duration of toxicity is in turn dependent upon the variable duration and growth of the toxic algae and the frequency and duration of blue-green algae blooms in general. Some factors affecting the growth and toxin production for *A. flos-aquae* cultures have been described (Gorham, *et al.*, 1964) but little information has been published on factors affecting toxicity. Hammer (1964) provides some information on bloom succession conditions for *A. flos-aquae* in a number of Saskatchewan lakes. It tends to occur in two major pulses, one in late spring when water temperature ranges from 14^{o}C upwards, and again in late summer, from August to October, after maximum temperatures have occurred. He found that blooms persisted from one week to two months. Blooms also seemed to appear one to two weeks after phosphate peaks. Since growth and toxin production by toxic strains are interrelated it can be expected that the incidence of algal poisonings will be greatest when maximum growth and

and concentration of colonies is occurring. All records of *A. flos-aquae* poisonings tend to confirm this.

Since mineral nutrition is important to growth it was reasonable to expect that it would be indirectly important to toxicity as well. It was also thought possible that it might be directly important to toxin production and variability, especially for the VFDF toxin, which is an alkaloid. In testing the possible effects of mineral elements on growth and toxin production by *A. flos-aquae* NRC-44-1 it would have been helpful to have some idea of their concentrations in natural waters. While concentrations of major elements, particularly nitrogen and phosphorus, are available for many natural waters, those of the minor elements are little known. Eyster (1967) has provided some information on the microinorganic requirements for growth of algae in general as well as the known enzyme reactions in which each element is important. Application to blue-green algae is difficult, however, since most work with minor elements has been done with green algae. Further complications arise because in artificial culture media in the presence of a strong chelator such as EDTA, the amounts of minor elements supplied may be 100 to 1000 times the amount of the free element required or found in natural waters where chelate concentrations are low.

Results of the mineral nutrient experiments did not reveal any large stimulation or suppression of toxicity caused by specific

elements. Deficiencies of sulfur or magnesium resulted in reduced growth but increased specific toxicity while deficiencies or excesses of minor elements resulted in reduced growth and specific toxicity. This indicates that growth and toxin production can be separated as partially independent processes. It appears that only great differences in the nutrient levels of water, sufficient to inhibit growth of *A. flos-aquae*, are likely to affect toxicity of blooms.

Oxygen tension was found not to stimulate VFDF production in *A. flos-aquae*. Low oxygen tensions, such as occur in decomposing blooms, tended to reduce rather than increase specific toxicity in nonaxenic cultures. Since low oxygen tensions often cause leakage of cell contents through increased permeability it suggests that VFDF may have been lost into the culture medium where it could be more easily destroyed by the bacteria present. Results did not indicate that products released in this way had any effect on toxicity.

As noted earlier by Peary and Gorham, aeration of shaken cultures causes a significant increase in biomass and toxicity but closer examination reveals that specific toxicity is not affected. Evidently the amount of CO_2 exchange to the buffered alkaline medium in the nonaerated shake flasks was insufficient to satisfy the needs of the alga for photosynthesis. Addition of carbonates or bicarbonates to the ASM-1-TR medium should promote better yields in terms of biomass and VFDF. Lange (1971)

has suggested that next to phosphorus and nitrogen, carbon dioxide may be one of the important limiting factors in bloom production by blue-green algae. He also suggests that bacterial decomposition of organic substances may be the source of carbon dioxide for stimulation of algal growth. In nature, therefore, the amount of carbon dioxide available during bloom development may indirectly affect the amount of toxin as blooms accumulate, with subsequent decomposition and release of VFDF near the shore.

The similarity between blue-green algae and bacteria are well known and have been discussed by Echlin and Morris (1965) and more recently by Stanier, *et al.* (1971). This similarity includes both physiological and structural aspects and because of this, attempts to separate the algae from the bacteria have often failed. Most of the bacterial contaminants of *A. flos-aquae* NRC-44-1 were gram-negative rods with and without active motility. The low-level contaminants were hardest to remove by the washing and plating procedure. The dark phenol treatment proved important in giving a greater algae-bacteria ratio for final separation of these low-level contaminants. Phenol is a surface active agent that acts on the cell membrane. Since the cell wall and membrane of algae and bacteria are similar it is necessary to obtain a greater degree of activity by the phenol for the bacteria. It was thought that algal cells with their metabolic rate lowered by storage in the dark prior to phenol treatment would make them less susceptible to phenol. The

heterotrophic bacteria would still have high metabolic activity particularly when organic additives are included in the medium and should be more susceptible to the phenol. When this dark phenol treatment was applied to NRC-44-1 a selective killing of the *Cytophaga* sp. contaminant occurred, which made possible its separation from the algae.

In trying to modify the procedure for washing and plating to find the right combination for growth of algae and bacteria, two helpful bacterial growth factors were found. When the wash and pour-plate technique first failed to work it was thought that the temperature of the agar was too high causing lysis of the added algal filaments. Two components were tried that would gel at a lower point than 1.5% agar (about 30°C). These were gelatin and sodium carboxymethylcellulose (CMC) (Furia, 1968). Both of these additives allowed lower pour temperatures but inhibited algal growth. They promoted good bacterial growth, however, with CMC supporting cellulose degraders, and were thus incorporated for bacteriological test media. As mentioned earlier, 1.0% agar was finally used for pours. This allowed pour temperatures of about 27 to 28°C which were better tolerated by the algae than 29 to 31°C. At this agar concentration, porosity of the gelled agar might be of concern in that it could allow movement of flagellated bacteria. Weir (1967) makes reference to agar strength and porosity. He mentions that the average pore diameter of a 2% agar

gel is about 3 nm. In reference to most immunoreactants (antibodies and antigens) of moderate molecular size, an agar gel of 0.3 to 1.5% would not inhibit free diffusion. The sizes of bacteria encountered in the algal cultures were not smaller than about 0.5 μ in diameter. Pore size of a 1.0% agar gel should thus inhibit passage of the bacteria.

To obtain an axenic culture of *A. flos-aquae* proved to be difficult. The two methods used for purity checks, phase contrast microscopy and growth with various organic nutrients, have limitations. With phase contrast observation it was necessary to check old cultures and to observe several fields. With biological test media, tests had to be made at various culture ages and at varying temperatures, with media being chosen that would grow a wide range of organisms.

It is expected that the method used to repurify NRC-44-1 can be repeated with other strains of planktonic blue-green algae. Slight modifications in mineral media or organic additives may be necessary to enhance particular algae or bacteria.

Repurification of NRC-44-1 to bacteria-specific and axenic conditions gave a much higher specific toxicity than non-axenic cultures grown under the same physical conditions. Biomass was not greatly affected so what was indicated was bacterial degradation or detoxification of some of the VFDF, probably the released portion. The specific bacteria capable of detoxification have not been isolated or identified. They are probably a com-

ponent of many if not all natural waterbloom floras, although it has yet to be proved. Their numbers probably increase along with the general increase of bacteria during bloom accumulation and decomposition and they may be another important factor affecting the variability of toxicity during a toxic bloom. The fact that they can account for variable toxicity in laboratory cultures, particularly those mass-produced in large volumes, will warrent further research into their identity, numbers and occurrence in toxic and nontoxic waterblooms.

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APPENDIX I

Components of ASM-1-TR Culture Solution

Component	Concentration, mM, in culture solution
$NaNO_3$	2.0
K_2HPO_4	0.1
$Na_2HPO_4 \cdot 7H_2O$	0.1
$MgCl_2 \cdot 6H_2O$	0.2
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.2
$FeCl_3$	0.004
Micronutrients:	
H_3BO_3	0.040
$MnCl_2$	0.007
$ZnCl_2$	0.0032
$CoCl_2$	0.00008
$CuCl_2$	0.0000008
Tricine	0.15
Na_2EDTA	0.02

APPENDIX II

Weight of Salts in ASM-1-TR

Component	mg/liter
$NaNO_3$	170.00
K_2HPO_4	17.40
Na_2HPO_4	14.20
$MgCl_2$	19.02
$MgSO_4$	24.08
$CaCl_2$	22.20
$FeCl_3$	0.65
H_3BO_3	2.47
$MnCl_2$	0.87
$ZnCl_2$	0.44
$CoCl_2$	0.01
$CuCl_2$	0.0001
Tricine	26.90
Na_2EDTA	6.64
TOTAL	304.88

APPENDIX III

*Components of ASM-2-TR Culture Solution

Include the same components of ASM-1-TR (Appendix I) plus peptones and vitamins as listed below:

Peptones:

Na Caseinate	250 mg
Bacto Tryptone	250 mg
Proteose Peptone	250 mg

Add above to 100 ml deionized water

Use 4 ml per liter for ASM-2-TR culture solution

Vitamins:

A-Working Solution

Thiamine (Vit. B_1)	10.0 mg
Cobalamine (Vit. B_{12})	.05 mg
Biotin	.50 mg

Add above to 100 ml deionized water

Use 2 ml per liter for ASM-2-TR culture solution

B-Working Solution

Riboflavin (Vit. B_2)	1.0 mg
Pyridoxine (Vit. B_6)	8.0 mg
Ca Pantothenate	4.0 mg
Folic Acid	0.1 mg

Add above to 100 ml deionized water

Use 2 ml per liter for ASM-2-TR culture solution

*NOTE: All concentrations in culture solution should be doubled when mixed with equal volumes of 2% agar gel.

APPENDIX IV

Components of Biological Test Media Used in Purification Technique

1. Gelatin - Vitamin - Peptone Medium

   Gelatin 12% (Difco); to this add vitamins and peptones as in ASM-2.

2. Sodium Caseinate Agar (freshwater bacteria)[1]

Peptone (B.D.H.)	0.5 g
Na Caseinate	0.5 g
Soluble Starch	0.5 g
Glycerol	1.0 ml
K_2HPO_4	0.2 g
$MgSO_4$	0.05 g
$FeCl_3$	"Trace"
Agar (Difco)	15.0 g
Distilled Water	1.0 liter

[1]Taylor, C. B. 1940. J. Hygiene, 40:616.

3. Soil Extract-Semisolid Medium[2]

K_2HPO_4	0.2 g
Yeast Extract (Difco)	1.0 g
Agar	3.0 g
Soil Extract	1.0 liter

   Autoclave 1 kg field soil and 1 liter of tap water
   Filter after adding small amount $CaSO_4$ and make up supernatent to 1 liter.

[2]Lochhead, A. G. and Margaret O. Burton. 1955. Can. J. Microbiol., 1:319.

APPENDIX IV (continued)

4. Casitone Agar

Add everything as in sodium caseinate agar except substitute 0.5 g casitone (Difco) for sodium caseinate.

Use of casitone is taken from Daft and Stewart (1971).

APPENDIX V

TABLE A

The effect of varying the concentration of the major elements in ASM-1-TR on growth and toxicity of nonaxenic *A. flos-aquae* NRC-44-1.

Element	Concentration, mM	Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
NO_3 as $NaNO_3$ (control 2.0 mM)	0.0	10	.015	320	139	4	17	4.3
	1.0	10	.075	240	239	19	40	2.1
	2.0	10	.115	120	334	29	111	3.8
	4.0	10	.145	240	511	36	85	2.4
	0.0	15	.099	240	160	25	27	1.1
	1.0	15	.110	160	248	28	62	2.2
	2.0	15	.430	60	413	108	275	2.5
	4.0	15	.412	120	578	103	193	1.9
	0.0	20	.095	120	159	24	53	2.2
	1.0	20	.195	120	269	49	90	1.8
	2.0	20	.551	60	443	138	295	2.1
	4.0	20	.573	80	619	144	310	2.2
Repeat	0.0	15	.080	320	155	20	19	1.0
	1.0	15	.105	160	247	27	62	2.3
	2.0	15	.095	240	329	24	55	2.3
	6.0	15	.095	480	669	24	56	2.3
	8.0	15	.085	960	836	21	35	1.7
	12.0	15	.070	NT*	1173	18	---	---

*Nontoxic

APPENDIX V, TABLE A (continued)

Element	Concentration, mM	Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
PO_4 as Na_2HPO_4 and K_2HPO_4 (control 0.10 mM)	0.00	10	.060	240	307	16	51	3.2
	0.05	10	.080	240	325	20	54	2.7
	0.10	10	.070	320	323	18	40	2.2
	0.20	10	.080	320	339	20	42	2.1
	0.00	15	.071	240	309	18	52	2.9
	0.05	15	.069	320	323	18	40	2.2
	0.10	15	.072	320	323	18	40	2.2
	0.20	15	.072	320	337	18	42	2.3
	0.00	20	.105	320	317	26	40	1.5
	0.05	20	.125	320	336	31	42	1.4
	0.10	20	.109	320	332	27	42	1.6
	0.20	20	.178	320	363	44	45	1.0
K as K_2HPO_4 and K_2NO_3 (control 0.1 mM)	0.0	10	.071	320	323	18	40	2.2
	0.1	10	.072	320	323	18	40	2.2
	0.5	10	.081	480	381	20	32	1.6
	1.0	10	.073	480	450	19	38	2.0
	0.0	15	.101	640	331	26	21	0.8
	0.1	15	.113	640	334	29	21	0.7
	0.5	15	.113	640	390	29	24	0.8
	1.0	15	.101	480	457	26	38	1.5
	0.0	20	.114	480	334	29	28	1.0
	0.1	20	.145	480	341	36	28	0.8
	0.5	20	.150	480	399	38	33	0.9
	1.0	20	.135	480	465	34	39	1.1

APPENDIX V, TABLE A (continued)

Element	Concentration, mM	Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
SO_4 as $MgSO_4$ and Na_2SO_4 (control 0.2 mM)	0.0	10	.038	480	315	10	26	2.6
	0.2	10	.055	320	319	14	40	2.9
	0.8	10	.050	640	396	13	25	1.9
	1.4	10	.050	640	474	13	30	2.3
	0.0	15	.045	640	314	11	20	1.8
	0.2	15	.055	640	319	14	20	1.4
	0.8	15	.114	640	412	29	26	0.9
	1.4	15	.105	640	487	26	30	1.2
	0.0	20	.040	640	315	10	20	2.0
	0.2	20	.113	480	334	29	28	1.0
	0.8	20	.098	640	408	25	26	1.0
	1.4	20	.123	640	492	31	31	1.0
Mg as $MgSO_4$ and $MgCl_2$ (control 0.4 mM)	0.0	10	.016	800	290	4	14	3.5
	0.4	10	.052	480	318	13	26	2.0
	0.8	10	.031	640	332	8	21	2.6
	1.2	10	.046	480	354	11	30	2.7
	0.0	15	.035	640	295	9	18	2.0
	0.4	15	.062	480	321	16	27	1.7
	0.8	15	.058	640	339	15	21	1.4
	1.2	15	.073	480	362	19	30	1.6
	0.0	20	.036	640	295	9	18	2.0
	0.4	20	.082	480	325	20	27	1.4
	0.8	20	.081	480	344	20	29	1.5
	1.2	20	.098	320	368	25	46	1.8

APPENDIX V, TABLE A (continued)

Element	Concentration, mM	Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
Ca as $CaCl_2$ (control 0.2 mM)	0.0	10	.035	800	292	9	15	1.7
	0.2	10	.075	320	324	19	40	2.1
	0.6	10	.083	320	370	21	46	2.2
	0.0	15	.045	960	294	11	12	1.1
	0.2	15	.090	320	328	23	41	1.8
	0.6	15	.105	320	375	26	47	1.8
	0.0	20	.070	480	301	18	25	1.4
	0.2	20	.115	320	334	29	42	1.4
	0.6	20	.115	320	378	29	47	1.6

APPENDIX V

TABLE B

The effect of varying the concentrations of Fe, Mn, Zn, B and Na_2EDTA on growth and toxicity of nonaxenic *A. flos-aquae* NRC-44-1.

Element	Concentration, mM	Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
Fe as $FeCl_3$ (control 0.004 mM)	0.000	10	.059	320	320	15	40	2.7
	0.004	10	.083	320	326	21	41	2.0
	0.006	10	.094	320	329	24	41	1.7
	0.008	10	.112	320	334	28	42	1.5
	0.000	15	.118	240	335	30	56	1.9
	0.004	15	.125	320	336	31	42	1.4
	0.006	15	.115	320	334	29	42	1.4
	0.008	15	.119	320	336	30	42	1.4
	0.000	20	.105	400	331	26	33	1.3
	0.004	20	.095	400	329	24	33	1.4
	0.006	20	.098	400	330	25	33	1.3
	0.008	20	.112	400	334	28	33	1.2
Repeat	0.000	15	.084	480	326	21	27	1.3
	0.002	15	.098	640	330	25	21	0.9
	0.004	15	.105	240	332	27	55	2.0
	0.016	15	.121	480	335	30	28	0.9
	0.024	15	.114	320	337	29	42	1.4
	0.040	15	.098	480	335	25	28	1.1

APPENDIX V, TABLE B (continued)

Element	Concentration, mM	Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
Mn as $MnCl_2$ (control 0.007 mM)	0.000	10	.049	480	316	12	26	2.2
	0.007	10	.070	320	323	18	40	2.2
	0.028	10	.060	480	324	16	27	1.7
	0.000	15	.041	480	314	10	26	2.6
	0.007	15	.055	480	319	14	26	1.9
	0.028	15	.024	480	315	7	26	3.7
Repeat	0.000	15	.075	320	323	19	40	2.1
	0.0035	15	.074	320	323	19	40	2.1
	0.007	15	.095	240	329	24	55	2.3
	0.014	15	.074	320	324	19	40	2.1
	0.021	15	.075	320	325	19	41	2.2
	0.042	15	.100	320	336	26	42	1.6
	0.070	15	.082	480	335	21	28	1.3
Zn as $ZnCl_2$ (control 0.0032 mM)	0.0000	15	.080	320	325	20	41	2.1
	0.0016	15	.075	240	324	19	54	2.8
	0.0032	15	.095	240	329	24	55	2.3
	0.0064	15	.065	320	322	17	40	2.4
	0.0096	15	.075	480	324	19	27	1.4
	0.0192	15	.045	480	320	12	27	2.3
	0.0320	15	.005	NT*	312	2	---	---

*Nontoxic

APPENDIX V, TABLE B (continued)

Element	Concentration, mM	Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
B (control 0.04 mM)	0.00	15	.110	320	331	28	41	1.5
	0.02	15	.112	320	333	29	42	1.4
	0.04	15	.105	240	332	27	55	2.0
	0.40	15	.105	480	346	27	29	1.1
	1.20	15	.096	480	367	24	30	1.3
Na_2EDTA (control 0.02 mM)	0.00	15	.013	NT*	301	3	---	---
	0.02	15	.075	320	324	19	41	2.2
	0.06	15	.082	480	340	21	28	1.3
	0.16	15	.085	480	376	22	31	1.5

*Nontoxic

APPENDIX V

TABLE C

The effect of varying the concentrations of some groups of minor elements and the chelator, relative to their concentration in ASM-1-TR, on growth and toxicity of nonaxenic *A. flos-aquae* NRC-44-1.

Elements	Concentration relative to control	Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
All minors (control as in ASM-1-TR)	0	10	.065	320	317	16	40	2.5
	1	10	.057	480	320	15	27	1.8
	10	10	.006	NT*	343	2	---	---
	16	10	.006	NT	367	2	---	---
	0	15	.119	320	331	30	41	1.4
	1	15	.114	320	334	29	42	1.4
	10	15	.010	NT	344	3	---	---
	16	15	.010	NT	368	3	---	---
	0	20	.144	320	337	36	42	1.2
	1	20	.154	480	344	39	29	0.7
	10	20	.010	NT	344	3	---	---
	16	20	.008	NT	367	2	---	---
Repeat	0	15	.055	640	314	13	20	1.5
	1	15	.095	240	327	22	55	2.5
	3	15	.085	960	334	21	14	0.7
	6	15	.025	NT	330	7	---	---
	10	15	.005	NT	343	2	---	---

*Nontoxic

APPENDIX V, TABLE C (continued)

Elements	Concentration relative to control	Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
All minors, Repeat, Aerated (control as in ASM-1-TR)	0	15	.240	120	361	60	120	2.0
	1	15	.580	60	560	255	374	1.5
	3	15	.700	80	636	323	318	0.9
	6	15	.035	NT*	334	9	---	---
	10	15	.020	NT	347	6	---	---
Mn, Zn (control as in ASM-1-TR)	0	15	.085	480	323	22	27	1.2
	1	15	.112	320	333	28	42	1.5
	3	15	.092	640	331	24	21	0.8
	6	15	.017	NT	315	5	---	---
	10	15	.005	NT	316	2	---	---
Increasing amounts of Mn, Zn and Na_2EDTA (control as in ASM-1-TR)	0 (Mn) 0 (Zn) 1 (EDTA)	15	.095	480	328	24	27	1.1
	1 1 1	15	.105	320	332	27	42	1.5
	3 3 3	15	.102	240	333	26	56	2.2
	6 6 6	15	.102	480	336	26	28	1.1
	10 10 8	15	.105	480	341	27	28	1.0

*Nontoxic

APPENDIX V, TABLE C (continued)

Elements	Concentration relative to control		Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
Increasing amounts of Mn and Na_2EDTA (control as in ASM-1-TR)	0	(Mn)							
	1	(EDTA)	15	.125	640	335	31	21	0.7
	1								
	1		15	.105	320	332	27	42	1.6
	3								
	3		15	.100	240	332	25	55	2.2
	6								
	6		15	.095	480	334	24	28	1.2
	10								
	8		15	.110	640	342	28	21	0.8
Increasing amounts of Zn and Na_2EDTA (control as in ASM-1-TR)	0	(Zn)							
	1	(EDTA)	15	.055	1280	320	15	10	0.7
	1								
	1		15	.105	320	332	27	42	1.6
	3								
	3		15	.100	240	330	25	55	2.2
	6								
	6		15	.137	240	340	35	57	1.6
	10								
	8		15	.120	480	335	30	28	0.9

APPENDIX V, TABLE C (continued)

Elements	Concentration relative to control	Culture Age, Days	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
B, Co, Cu (control as in ASM-1-TR)	0	15	.122	320	331	28	41	1.5
	1	15	.168	240	347	42	58	1.4
	3	15	.145	320	345	36	43	1.2
	6	15	.123	240	346	31	58	1.9
	10	15	.150	240	361	38	60	1.6

APPENDIX V

TABLE D

The effect of varying some components of ASM-1-TR on growth and toxicity of 15-day old cultures of bacteria-specific *A. flos-aquae* NRC-44-1.

Element	Concentration, mM	OD, 750 nm	MLD, mg/kg	Dry wt. per liter Biomass + Salts, mg	Dry wt. per liter Biomass, mg	Mouse Units per liter Biomass + Salts	Mouse Units per mg Biomass
NO_3 as $NaNO_3$ (control 2.0 mM)	0.0	.082	160	156	21	39	1.9
	1.0	.082	160	241	21	60	2.9
	2.0	.075	240	324	19	54	2.8
	6.0	.085	480	667	22	55	2.5
Mn as $MnCl_2$ (control 0.007 mM)	0.000	.019	480	310	5	26	5.2
	0.007	.075	240	324	19	54	2.8
	0.042	.120	160	340	30	85	2.8
	0.070	.120	240	345	30	57	1.9
Zn as $ZnCl_2$ (control 0.0032 mM)	0.0000	.075	320	324	19	40	2.1
	0.0032	.075	240	324	19	54	2.8
	0.0192	.035	320	316	9	40	4.4
	0.0320	.000	NT*	---	--	--	---
Fe as $FeCl_3$ (control 0.004 mM)	0.000	.065	480	332	17	28	1.6
	0.002	.025	480	312	7	26	3.7
	0.004	.075	240	324	19	54	2.8
	0.024	.030	480	314	8	26	3.3
	0.040	.050	480	323	13	27	2.1

*Nontoxic